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THE INFLUENCE OF ANALOGUES OF 1-FLUORO-2,4-DINITROBENZENE ON THE KINETICS OF IRREVERSIBLE INHIBITION OF SUGAR TRANSPORT IN THE HUMAN ERYTHROCYTE

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SUMMARY

1. The effect of analogues of 1-fluoro-2,4-dinitrobenzene on the kinetics of inhibition of the human erythrocyte transport system has been investigated.

2. The analogues increase the rate of inhibition of glucose transport and modify the kinetics to a pseudo-unimolecular form in the presence of inhibitor.

3. A mechanism for inhibition of the transport system by 1-fluoro-2,4-dinitrobenzene is proposed.

INTRODUCTION

Several models have been proposed for the 'carriers' which mediate the movements of specific nonelectrolytes into and out of cells (see refs. 1–3). Certain of these models (reviewed by STEIN[4]) consist of a protein unit or units, plugged on, in or through the membrane, with which the substrate molecule combines (with enzyme-type specificity) before it is carried across the membrane.

The glucose carrier system of the human erythrocyte has been shown by BOWYER AND WIDDAS[5] to be inhibited by the protein end group reagent 1-fluoro-2,4-dinitrobenzene (FDNB) with kinetics second order with respect to FDNB concentration with a high Q_{10} value of 7 at 25°. These findings suggest that the chemical reaction of the irreversible inhibition step, between the active site (or a site associated with, but not itself, the carrier active site) and FDNB, is complex, since reactions involving other proteins of the membrane and FDNB are first order with respect to both reactants[6]. GREEN[7], in a study of FDNB reaction with bovine serum albumin, noted the presence of several 'high reactivity' groups in the protein, whose high reaction rates with FDNB were caused by hydrophobic interaction between FDNB and portions of the protein and suppressed in the presence of hydrophobic agents. It is evident that the carrier active centre is also in an unusual condition. This could be due to either the intrinsic structure of the carrier or the presence of the membrane around the carrier which could, possibly, confer by hydrophobic interaction properties on the protein not observed in aqueous solution or suspension.

Abbreviations: FDNB, 1-fluoro-2,4-dinitrobenzene; CDNB, 1-chloro-2,4-dinitrobenzene; DNB, 2,4-dinitrobenzene.

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To explain the second-order kinetics of FDNB inhibiting the glucose carrier, one might, therefore, suggest that two molecules of FDNB act with one glucose carrier unit, with facilitation of attack by a second FDNB molecule following initial binding of a first molecule of FDNB. The question arises can analogues of FDNB replace an FDNB molecule in this co-operative attack and it is this question which the present paper attempts to answer.

The proportion of sugar carrier sites remaining after treatment of erythrocytes with mixtures of FDNB and 2,4-dinitrobenzene (DNB), and FDNB and 1-chloro-2,4-dinitrobenzene (CDNB), was estimated by an assay of sorbose permeability (sorbose being carried by the same transport system as glucose).

MATERIALS

Human erythrocytes were obtained from outdated transfusion blood stored in acid–citrate–dextrose medium at 4°. On average the cells were used 3–4 weeks after removal from the donor. The cells were washed twice in isotonic phosphate–saline buffer (25 mM in phosphate and 1 % in NaCl (pH 7.4) at 37°, the plasma and leukocytes being removed as the top layer of cells after centrifugation and then stored overnight in phosphate–saline buffer at 4°. Before use the cells were washed once and made to 50 % haematocrit in the phosphate–saline medium. The radioactive sugar used was L-[^{14}C]sorbose (Radiochemical Centre). The unlabelled sugar was L (–)-sorbose (British Drug Houses) and the inhibitory agents FDNB 1-fluoro-2,4-dinitrobenzene (British Drug Houses), DNB (Hopkin and Williams) and CDNB (British Drug Houses).

METHODS

The inhibitors were added in ethanol to phosphate–saline buffer (pH 7.4, 25°) 15 sec before addition of the erythrocyte suspension and mixed to eliminate emulsion of inhibitor ethanolic solution. The incubation mixture was 4 % in ethanol at about 16 % haematocrit and maintained at 25° with shaking. (Control samples were 4 % in ethanol without the inhibitor.) Incubation was stopped after 30 min by a 20-fold dilution with ice-cold phosphate–saline medium. The erythrocytes were then washed twice and resuspended in the medium for sorbose permeability assay, at about 25 % haematocrit (the time elapsed between inhibition incubation and assay was 1 h). For the assay, ^{14}C-labelled sorbose solution (5 mM final concn.) was added to the cell suspension in duplicate samples and the translocation stopped after 5 min of shaking at 25°, by addition of 6 vol. ice-cold 'stopper' (4 mM $HgCl_2$, 1.25 mM KI, 1 % NaCl) (modified from LEVINE AND STEIN[8]). The cells were then centrifuged down, the supernatant removed and the interior of the tube wiped with tissue. The amount of sorbose trapped in the extracellular space of the cell pellet was estimated by mixing the sorbose with the stopping solution before addition to the cell suspension.

The sorbose of the cell pellet was estimated by extraction in trichloroacetic acid (10 %) and a tracer assay of aliquots of the trichloroacetic acid extract in the Packard Tricarb liquid scintillation counter using 0.4 % 2,5-diphenyloxazole and 0.04 % dimethyl-1,4-bis-2-(5-phenyloxazolyl)benzene in a xylene base, diluted with absolute

ethanol (68:30, v/v). The degree of quenching was found, by use of an external standard, to be constant from sample to sample.

It was found that this method allowed determination of the level of inhibition with a standard deviation of 1.9%.

RESULTS

Fig. 1 shows the dependence of the degree of inhibition of sorbose uptake on the composition of a DNB and FDNB system (total concentration of FDNB and DNB 2 mM in all cases). The inhibition due to FDNB at the same concentration levels with the omission of DNB was computed as the 'theoretical line' in the figure, on the basis of experimental determination of the degree of inhibition against FDNB concentration. DNB itself was found not to have any detectable inhibitory effect at 2 mM concentration. The difference curve in Fig. 1 illustrates that excess inhibition results in the presence of DNB, over and above that due to FDNB alone.

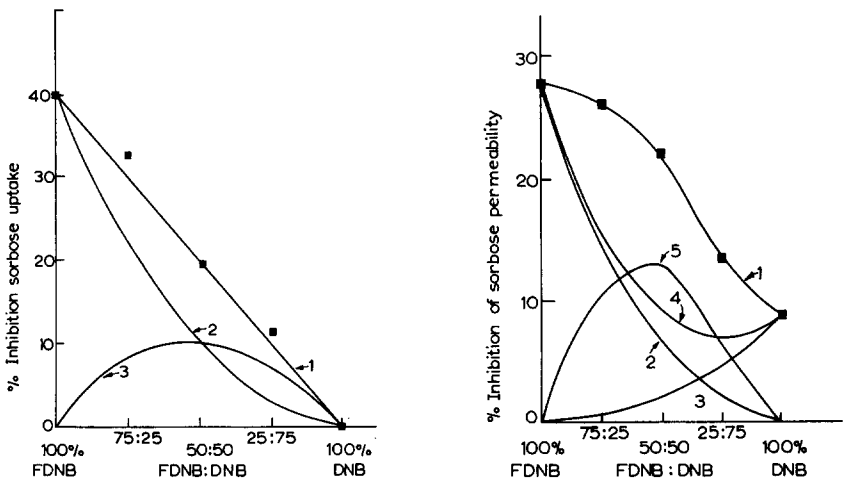

Fig. 1. Inhibition of sorbose uptake by mixtures of DNB and FDNB. Curve 1, experimental results; curve 2, result for FDNB at same concentrations when DNB absent; Curve 3, excess inhibition.

Fig. 2. Inhibition of sorbose uptake by mixtures of CDNB and FDNB. Curve 1, experimental results; Curve 2, result for FDNB at same concentrations when CDNB absent; Curve 3, result for CDNB at same concentrations when FDNB absent; Curve 4, theoretical result if no interaction between FDNB and CDNB; Curve 5, excess inhibition.

The kinetics of inhibition due to FDNB (in the presence of DNB) are now seen to be first order with respect to FDNB concentration. The symmetrical shape of the difference curve suggests that FDNB and DNB are equally effective in co-operating with FDNB in the carrier inactivation reaction. Fig. 2 shows similar results obtained using CDNB and FDNB (total concentration 1.8 mM). In this case the CDNB has a significant inhibitory effect on the uptake of sorbose into the human erythrocyte at the level used – its kinetics have been found like FDNB to be dependent on the square of the concentration in the incubation reaction. Fig. 3 illustrates the inhibition of sorbose uptake under conditions where the product of

time of inhibition incubation and CDNB concentration is held constant for all points (30 mM·min). Under such conditions the linear result shows inhibition to be square dependent according to the equation $I = ktc^2$ (k = constant, t = time, c = concentration of inhibitor and I = percent inhibition of sorbose uptake).

Fig. 4 illustrates an experiment to determine whether the co-operation between FDNB and DNB in the inhibition of sorbose uptake is due to initial binding of DNB followed by facilitated FDNB attack on the glucose transfer system or interaction to modify the kinetics of the inhibition reaction. DNB and FDNB were incubated with erythrocytes either consecutively or simultaneously. The cells were initially incubated (30 min at 25°) with DNB (0.5–3.0 mM) or an ethanol control. After washing the cells were incubated with FDNB (1 mM) or FDNB (1 mM) and DNB (0.5–3.0 mM), respectively (30 min at 25°). The results show that an initial reaction between DNB and the protein does not occur and that co-operation between FDNB and DNB takes place during the reaction between FDNB and the glucose transfer system.

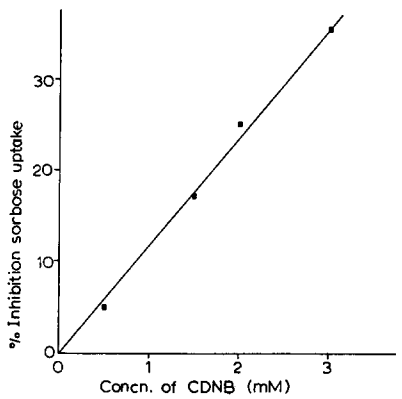

Fig. 3. Inhibition of sorbose uptake by CDNB under conditions to illustrate square-dependent kinetics of inhibition (product of concentration CDNB and time of incubation held constant at 30 mM·min).

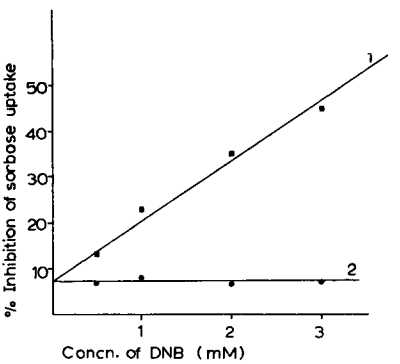

Fig. 4. Inhibition of sorbose uptake caused by 1 mM FDNB when erythrocytes incubated simultaneously with DNB (Curve 1), and when preincubated with DNB (Curve 2).

DISCUSSION

Figs. 1 and 2 show that analogues of FDNB can 'co-operate' with FDNB to modify the kinetics and reaction rates of the FDNB inhibition reaction. Since DNB has by itself no measurable reactivity with the carrier at the levels used, then it must either react with FDNB to form a complex inhibitor whose kinetics are being observed, or interact with one of a pair of binding sites in the inhibition step with FDNB. Both these possible postulates could explain the apparent 1:1 relationship of DNB: FDNB for maximal excess activity. We have observed, however, that the absorbance at 420 mμ of mixtures of FDNB and DNB increases linearly with relation to DNB, using a fixed level of FDNB, increasing the concentration of DNB to 3 times that of the FDNB concentration. The formation of a complex

between DNB and FDNB might be expected to be revealed in a change of slope of the absorbance curve.

Thus it seems that DNB or CDNB can replace a molecule of FDNB during the simultaneous attack of two FDNB molecules at each glucose carrier site. One might suggest that the replacing molecule while not chemically involved initially (DNB cannot act chemically or bind by itself) acts as a directing agent for FDNB which subsequently reacts with the glucose carrier to cause inhibition. The finding that FDNB and analogue molecules act pairwise in the inhibition reaction suggests that direct involvement with the active centre region of the protein and not a generalised hydrophobic effect is the basis of the co-operation found.

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